# DISCUSSION

Plants are compelled to withstand stresses of all kinds, be it biotic, abiotic or anthropogenic as a consequence of their immobility. Tea, being perennial, is subjected to varying environmental conditions throughout its life as well as to numerous attacks by pest and pathogens, which in turn are influenced by various environmental conditions. Root system the foundation of the plant is weakened by many primary rot pathogens. *Ustulina zonata*, one of the soil borne fungal pathogens causes charcoal stump rot, a primary root disease. The initial infection process involving recognition events between plants and fungal pathogens is essential for the establishment. In nature plants have evolved multicomponent coordinated mechanisms by which they can defend themselves against the multitude of organisms attacking them. The art and science of plant disease control has moved in the direction of biological control of plant pathogens, including use of introduced antagonists. It is now widely recognized that biological control of plant pathogen is a distinct possibility for the future and can be successfully exploited in modern agriculture, especially within framework of integrated disease management systems. Integrated control is a flexible, multi-dimensional approach to disease control utilizing a range of control components such as biological, cultural and chemical strategies needed to hold diseases below damaging economic threshold without damaging the agro-ecosystem.

The rationale behind the disease control is to check pathogen's growth in the host and improve the health status of the plant. Disease resistance and susceptibility in plants do not represent any absolute values. Even susceptible variety shows resistance to its pathogen under certain cropping conditions or by treating stress situation. This would suggest that even a susceptible variety has a potentially effective defense mechanism and that by manipulating cropping conditions or by treating stress, it may be possible to elicit the expression of such latent defense potential during host parasite interaction. This constitutes the very basis of induced resistance in plants as a possible disease control measure. Since the first report of induction of resistance in plants against their fungal pathogens by prior inoculation with their less virulent form, the emphasis had been mostly on biological induction of resistance. Considerable evidence has now accumulated to that prior inoculation of susceptible plant host with an avirulent form of pathogen. Cultivars of non pathogenic races of pathogens of both homologous and heterologous

nature or non pathogen can provide it significant levels of protection form the subsequent attack of the virulent forms of pathogen (Purkayastha, 1994; Mukhopaddhya, 1994). Plants so protected develop less disease symptoms. In some cases such induced or acquired resistance is systemic in nature and persists effectively over a fairly long periods. Even though effective field level protection has been achieved by this method against many diseases of Tobacco, Bean, Cucumber and Melons, various logistical problems relation to the large scale production of the inducer biotic agent and its application to the crop under field conditions make this approach both cumbersome and uneconomic an heavily limit its utility as a measure for plant disease control particularly in the Indian agricultural perspective.

In the present investigation, sixteen tea varieties were screened against *U. zonata*. Among all the tested varieties, BSS-2, BS/7A/76 and AV-2 were found to be highly susceptible, while UPASI-9, UPASI-2 and UPASI-3 were most resistant. Although less in known with certainty about the specific recognition events that predict incompatible host-pathogen interaction, considerable genetic and biochemical evidence indicates that constitutive specificity imparting molecules must exist in the incompatible antifungal compounds at the infection site. Cell recognition has been defined as the initial event of cell-cell communication which elicits morphological, physiological and biochemical response. Surface molecules of eukaryote cells have been involved in cell-cell recognition and/or adhesion and as receptors for various effects. Many of these specificity imparting molecules are glycoproteins, and fungi are known to possess them on their cell-walls and plasma membranes (Keen and Legrand, 1980; Ransom *et al.*, 1992).

In the initial stage of infection at the cellular level the exchange of molecular signals between host and parasite is considered to be one of the mechanisms resulting in the specificity of such interactions. The genetic information contained in nucleic acid is expressed in the cell via protein synthesis. Several proteins function as enzymes in the metabolic pathways, which synthesize or breakdown cellular components. When plants containing various kinds of proteins are infected by pathogens, the proteins in the penetrated plant cells are changed chemically and physically. Thus qualitative and

quantitative changes in proteins are related to both plant and pathogen. A protein competition model was proposed by Jones and Hartley (1999) for predicting total phenolic allocations and concentration in leaves of terrestrial higher plants. They suggested that protein and phenol synthesis compete for the common limiting resource-phenylalanine and hence protein and phenolic allocations are inversely correlated.

In the present investigation changes in the protein content was noted in the *U. zonata* inoculated leaves of susceptible varieties in relation to their healthy control. Increased protein level was also detected after infection of susceptible bean leaves by *Uromyces phaseoli.* The greater accumulation of protein in susceptible host after inoculation may also be attributed to the total proteins of both host and parasite. However, it is difficult to separate the relative contribution of host and parasite to the total protein content. It is evident from the above statement that some changes occur in proteins of infected plants. However, these changes are not always significant. Sometimes protein content of the host remains more or less similar even after inoculation but isozyme pattern may change.

Environmental effects in phenolics are all the more long-lasting, as they have to cope of such conditions year after year. In a similar study on tea with the fungus *Glomerella cingulata* which causes brown blight of tea, it was reported that high humidity and rainfall were the most important factors predisposing the plants to infection (Chakraborty *et. al.*, 2002). Phenols are also known to play definite roles in a plant defense. Considering this in the present stud phenol contents of the healthy and artificially inoculated (with *U. zonata*) plants were determined. It has been reported previously that quinones in plant tissues react with proteins to form melanin and other tannins leading to the discoloration of damaged tea leaves. Many studies have demonstrated the importance of phenolic compounds in plant defense. In general, plant phenolics have a diverse range of biological activity, depending on their structure, degree of polymerization; stereo isomeric differences etc. interaction between phenolic compounds and environmental conditions determines their action. Polyphenols have a distinctive ability to engage in molecular recognition, or formation of intermolecular complexes with each other and with other molecules (Haslam, 1999). In the present

*Pestalotiopsis theae, Glomerella cingulata*] has been described by Chakraborty *et. al.*, (1995). Biochemical responses to tea plants exposed to biotic stress due to blister blight infection caused by *Exobasidium vexans* in the levels of phenols and enzyme activities were studied (Sharma and Chakraborty, 2004).

In the present study, the levels of phenolics in leaves of resistant and susceptible tea varieties were estimated following inoculation with *U. zonata.* Host responses could be differentiated by changes in content of phenolic compounds. In both the cases total phenol and orthodihydroxy phenol content increased in resistant varieties but decreased in susceptible varieties in comparison to their healthy controls. Hammerschmidt and Nicholson (1977) demonstrated a clear difference between resistant and susceptible interaction of maize to *Colletotrichum graminicola* based on accumulation of phenols. Sridhar and Ou (1974) reported differences in total phenolics accumulation in the interaction of *Pyricularia oryzae* with rice. However, no differences were found in the phenolic content in the interaction of maize to *Colletotrichum graminicola* based on accumulation of phenols. On the other hand, a resistant cotton cultivar contained fairly high amount of total as well as orthodihydroxy phenol than susceptible cultivar. In the present study, greater accumulation of orthodihydroxy phenol in resistant interaction of *U. zonata* and tea varieties indicated that this might play a role in disease resistance mechanism. Orthodihydroxy phenols play a major role in disease resistance and disease development. They are easily oxidized to highly reactive quinones which are effective inhibitors of sulphydryl enzymes, thereby preventing the metabolic activities of host and parasite cells (Mansfield 2000). The UV spectra from both the healthy and *U. zonata* inoculated tea roots were analyzed at 290nm. A sharp peak at retention time 2.6 was present in both but in the healthy extracts the peak height was much smaller than the inoculated one. Other small humps and shoulders were also evident in both the cases.

It is known than catechin is oxidatively cleaved to some simpler phenols and phenolic acids like catechol, phloroglucinol and protocatechuic acid. Sambandam el. Al., (1982) isolated and enzyme (catechin 2-3 dioxygensis) from *Chaetomium cupreum* which cleaved catechin into simpler phenols. It is not unreasonable to speculate that the antifungal compound cleaved to some simpler phenols in the present study. In the susceptible variety, the breakdown of catechin was almost complete while traces were

evident in the resistant variety even after 48 h of inoculation. Accumulation of pyrocatechol in resistant varieties increased after 48 h of inoculation with *U. zonata.* Increased level of pyrocatechol may be associated with the differential host responses to disease production.

Accumulation of defense enzymes such as phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO), peroxidase (PO), in tea varieties following inoculation with *U. zonata* were determined. PPO usually accumulated following inoculation of plants. PPO transcript levels systemically increased in tomato when mature leaflets were injured. Increased activity of PPO and PO was demonstrated in the cucumber leaf in the vicinity of the lesions caused by some foliar pathogens. Among all the stress related enzymes, the role of peroxidase has been most thoroughly worked out. PO is a metallo-enzyme containing porphyrin bound iron. The enzyme acts on a wide range of substrates including phenols, aromatic amines, amino acids and inorganic compounds. These are ubiquitous to plants and are characterized by a large number of isozymes. Various naturally occurring and synthetic substances, growth regulator and environmental factors markedly influence the activity of these peroxidases. Increased PO activity in susceptible cultivars were noticed when compared with the resistant following treatement with sodium bisulphate prior to inoculation with *Botrytis maydis.* On the other hand, there are also reports of increased PO activity due to induction of resistance (Chakraborty *et. al.,* 2005). The induction of PO activity by pathogens and methyl jasmonate and existence of multiple molecular forms of peroxidase in tea has also been reported (Sharma and Chakraborty, 2004). Previous reports indicate that oxidative enzymes such as PPO and PO as well as those involved in phenolic biosnthesis such as PAL are involved in defense reactions in plants. Considering the importance of phenol metabolism in tea plants, those three enzymes (PAL, POX and PPO) were selected in the present investigation. An elevation in the level of activity of PAL has been frequently demonstrated to be one of the earliest responses of plants to biotic (Chakraborty *et. al.,* 1993) or to other environmental stresses. In sorghum, naturally occurring high levels of PAL activity induced by light should be differentiated from the activity induced as a response of attempted fungal infection. Bhattacharya and Ward (1987) reported that PAL activity in soybean was enhanced in the resistance response of soybean hypocotyls to *Phytophthora megasperma.* Considering that PAL is a key enzyme in the biosynthesis,

not only of phytoalexins, but also of phenolic compounds have been associated with resistance responses in various host plants, it may be suggested that activity of PAL could be useful indicators of the activation of defense related enzymes.

There is surface to surface interaction between the host and pathogen. Recognition or interaction as compatible depends on some kinds of molecular similarities, between the host and pathogen (Chakraborty, 1998; Chakraborty and Saha, 1994; Chakraborty *et al.* 1995, 2002). One of the prerequisites for the successful establishment of the pathogen in the host is based on close serological similarity between host and pathogen. This serological relationship between host and pathogen has been exploited for development of pathogen detection systems in the host using PAb raised against the pathogen. Such disease detection and diagnostic kits have the advantage over conventional methods by being specific and having the ability to detect even minute amounts in tissues. Commercial diagnostic kits have been offered in recent years for the rapid diagnosis of several fungi in plant tissues, soil and water (Werres and Steffens, 1994). Most common among these techniques are ELISA, Dot- blot, Western blot etc. used in large scale disease indexing programmes in perennial and vegetatively propagated crops (Clark, 1981). Timely detection of disease especially root diseases combined with proper management practices can lessen crop loss to a great extent.

Enzyme linked immunosorbent assay (ELISA) is probably one of the most sensitive serological techniques for the detection of pathogen in host tissues (Chakraborty and Chakraborty, 2003). In the present study polyclonal antibody was raised against mycelia and cell wall of *U. zonata*. The antisera obtained were purified to minimize non specific binding. At the beginning, the sensitivity of the assay was optimized. Root antigens of sixteen tea varieties, non host and non pathogen were cross reacted separately with PAb of *U. zonata*. Presence of cross reactive antigens (CRA) between *U. zonata* and tea varieties were evident in immunodiffusion test. However, weak precipitation reaction was observed with antigens of some selected tea varieties. No common antigenic substance was found between *U. zonata* and non host and non pathogen. The presence of CRA and their involvement in various host parasite combinations have been observed. These are cotton and *Fusarium oxysporum f. sp. vasinfectum* (Charudattan, 1970); cotton and *Verticillium alboatrum* (Charudattan and DeVay, 1972); sweet potato and *Ceratocystis fimbriatae* (DeVay *et. al.,* 1972); potato and *Phytophthora infestans*

(Palmerly and Callow, 1978, Alba and DeVay, 1985); soybean and *Macrophomina phaseolina* (Chakraborty and Purkayastha, 1983); coffee and *Hemilea vastatrix* (Alba *et. al.*, 1983); jute and *Colletotrichum corchori* Bhattacharya and Purkayastha, 1985); soybean and *Colletotrichum dematum* var. *truncate* ( Purkayastha and Banerjee, 1986), tea and *Bipolaris carbonum* (Chakraborty and Saha, 1994); tea and *Pestalotiopsis theae* (Chakraborty *et. al.*, 1995); soybean and *Fusarium oxysporum* (Chakraborty *et. al.*, 1997). tea and *Glomerella cingulata* (Chakraborty *et.al.*, 2002), in the present study PTA-ELISA readily detected CRA between tea root antigens and *U. zonata* at a concentration of 1:250 antiserum dilution. Alba and DeVay (1985) also detected CRA in crude preparations and in purified preparations from mycelia of *Phytophthora infestans* using antisera of two potato cultivars at concentrations lower than 50 µg/ml protein in indirect ELISA. Visible outcome of a compatible host pathogen interaction may be obtained in many cases only after few days of infection, by which time the pathogen would be well established in the host tissues. Various formats of ELISA using polyclonal antibody have found widespread application in plant pathology and are routinely used for detection and identification purposes (Lyons and White, 1992; Hansen and Wick, 1993, Chakraborty *et. al.*, 1995; 1996; Chakraborty *et. al.*, 2002).

It is also important in the studies on host parasite relationship to determine the cellular location of the pathogen. For this purpose in this study, indirect immunofluorescence tests were conducted with cross sections of healthy and artificially inoculated (with *U. zonata*) tea roots and mycelia. Transverse sections from infected roots were made and PAb raised against mycelial antigens of *U. zonata* were used for probing the fungal hyphae which penetrate the root tissues. Bright fluorescence was observed in the cross sections of tea roots. DeVay *et. al.*, (1981) determined the tissue and cellular location of major cross reactive antigens (CRA) shared by cotton and *F. oxysporum f. sp. vasinfectum.* Cellular location of CRA in tea leaf tissues shared by three foliar fungal pathogens such as *Bipolaris carbonum* (Chakraborty and Saha, 1994); *Pestalotionpsis theae* ( Chakraborty *et. al.* 1995) and *Exobasidium vexans* (Sharma and Chakraborty, 2004) have been demonstrated. Besides detection of pathogen in host tissues using antibody based immunofluorescent technique has been reported by several previous authors (Reddy and Ananthanarayan,1984) On the basis of immunofluorescence studies, Dewey *et. al.*, (1984) demonstrated the presence of mycelium and

chalmydospores in naturally and artificially infested soil samples, using this technique. Different test formats including indirect ELISA, western blotting, dot blot and indirect immunofluorescence was assessed for their potential to detect resting spores of *Plasmodiophora brassica (* Wakeham and White 1996) as well as *Fomes lamaoensis* (Chakraborty *et. al.*, 2002) in soil.

The dot immunobinding assay was developed using alkaline phosphates substrate 5-bromo-4chloroindolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) to detect the precipitated hydroxyl group. When the substrate 5-bromo-4chloroindolyl phosphate is used, the phosphate is cleaved by the enzyme and the hydoxyl group precipitates. The hydroxyl group then tautomerizes forming a ketone and under alkaline conditions dimerization occurs, forming a dehydroindigo. In the process of dimerizing, it releases hydrogen ions and reduces the nitroblue tetrazolium which precipitates, forming an intense blue deposition of diformazan. The dot immunobinding technique has also been found to be a rapid and sensitive method for detection of fungal pathogens. In the present study, antigens were prepared from charcoal stump rot infected tea roots. Naturally infected tea root as well as root artificially inoculated with *U. zonata* were tested on nitrocellulose paper. Infected and artificially inoculated root antigens gave intense dots when compared to the healthy control confirming the presence of fungal pathogens. So, early detection of disease is an important requisite for development of management strategies. A microtitre immunospore trapping device, which uses a suction system to trap air particulates directly by impaction into microtitre wells, has been used successfully for the rapid immunodetection and quantification of ascospores of *Mycosphaerella brassicicola* and conidia of *Botrytis cinerea* ( Kennedy *et. al.*, 2000).

Plants have well developed defense mechanisms which enable them to defend themselves against parasites in their tissues. The biochemical basis for this resistance against microbial attack consists of both preformed and post-infectional ones. Preformed defenses are often regarded as general or unspecific as compared to inducible defense systems which are highly specific. Though the versatile multicomponent defense is adequate to provide them protection against most of their potential pathogens, only a few of them can overcome this defense and cause disease. Varieties within the host species are resistant when they possess one or more resistant gene(s) and susceptible when they lack any such gene. To account for the observed specificity and degree of variability of

host parasite system, the fungal receptor must have high information content which involves recognition between the host and pathogen both at the cellular and subcellular level. A cell reacts in a special way as a consequence of an association with another cell because it acquires information, which is conveyed through chemical or physical signals in the process or recognition. The spatial and temporal deployment of plant defense responses involves the complex interplay of signal events, often resulting in superimposition of signaling processes. In spite of lacking immune responses like animals, plants have nevertheless evolved immune mechanisms of various types by which they can account for the advance of foreign organisms. The result is that disease tends to be specific, a given pathogen usually infecting a distinct range of host plants.

In the present investigation, using PTA-ELISA formats and PAb of *U. zonata*, treated and untreated plants exposed to natural inoculum after 15 and 30 days of soil amendments were compared. The absorbance ($A_{405}$) values were always reduced in treated root tissues than untreated ones. It indicates clearly that in the treated root tissues the establishment of the pathogen (*U. zonata*) was not successful due to the application of biocontrol agents. Detection of *U. zonata* in tea root tissues and rhizosphere soil of different treatment with pathogen and biocontrol agents was also determined immunologically in both root tissues and soil. For this purpose, PTA-ELISA format was carried out. Results showed that ELISA values of root tissues treated with *T. harzianum* and *T. viride* were significantly lesser than with *U. zonata* alone. The same trend of results was obtained in infested rhizosphere soil through PTA-ELISA analysis. This result is in conformity with that planting of tea seedlings after dipping roots in spore suspension of *T. harzianum* reduced 56.6% mortality of plant due to fungal infection. However the reduction of mortality of plant increased to 62.2% when *T. harzianum* was applied to soil. Significant control of charcoal stump rot of tea with antagonistic microflora and role of *T. hazianum* and *T. viride* as biocontrol agents have been well established. In the present study, antigens prepared from mycelia of *U. zonata* amended soils and following application of *T. harzianum* and *T. viride* were prepared and tested on nitrocellulose paper using PAbs raised against mycelia of *U. zonata* and NBT/BCIP as substrate. Antigens of homologous source showed deep colored dot when compared with soil antigens prepared from treated organic amendments. Other tea root pathogens responded slightly reactivity with *U. zonata.* Walsh *et. al.* (1996) also performed western

blotting using the crude serum of *Spongospora subterranean* spore balls. Different test formats including indirect ELISA, western blotting, dot blot and indirect immunofluorescence were assayed by Wakeham and White (1996) for detection of resting spores of *Plasmodiophora brasica* in soil. In conclusion, it can be stated that charcoal stump rot can cause severe damage to tea plants, as primary root disease and such immunodetection techniques makes it possible to detect micro quantities of the pathogen within root tissue and rhizosphere soil before much damage cause by the pathogen. Therefore, an accurate, rapid and cost-effective diagnosis and rapid detection of pathogen is important to take preventive steps for disease management.

Further in this investigation, effective integrated management practices against *U. zonata* were tested *in vivo*. Biocontrol agents [*T. harzianum, T.viride*] alone and in combination with neem cake, oil cake provided control of charcoal stump rot disease in all the three modes of application viz, simultaneous, repeated and post infection. But repeated application of neem cake, oil cake with various combinations of cow dung, rabbit manure and chicken manure were found to be significant. A possible long-term benefit of increased implementation of microbial control would be reduced input into agriculture, particularly if seasonal colonization and introduction-establishment come into widespread use. Initially, inputs due to implementation of microbial control are more likely to increase than decrease. Biological control using agriculturally important microorganisms is simply one of the best potential alternatives for disease control that could be made available in a relatively short time period. A successful disease control program depends on a crop production system which is closely aligned with the goals of disease management. In this context, attempts using biocontrol agents and organic residue materials as an integrated approach for disease control assumes much greater significance. There is potential for yield increase in the near future. Biomass production, their suitable formulation for commercialization of antagonists to check chemical fungicides usage needs to be developed.